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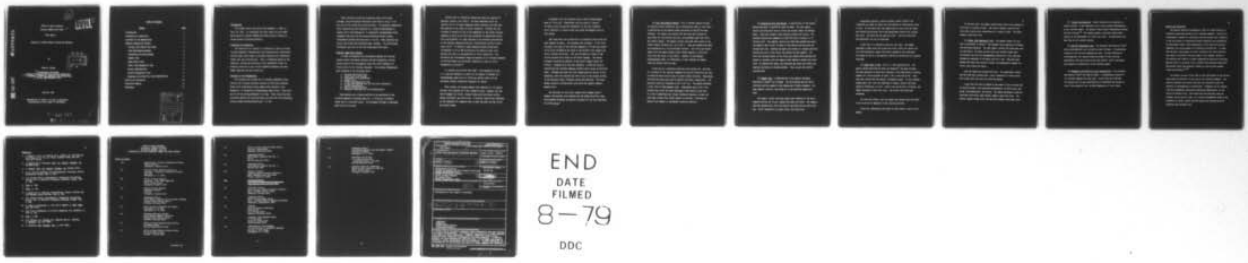

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Final Report

Studies on Plasma Soluble Fluorescent Melanins

by

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Introduction

This report covers the period from September 1, 1978, to April 30, 1979. It constitutes the final report for the eight-month period of Contract N00014-78-C-0766 entitled "Studies on Plasma Soluble Fluorescent Melanins.

Literature on Lipofuscins

The reason for our interest in lipofuscin is that our plasma isolated compounds (rheomelanins) have characteristic fluorescence (yellow-green) which seems to associate them with the lipofuscins rather than the solid melanins. Thus, a literature search on the chemistry, biology and structure of the lipofuscins as well as melanins, which are a part of the lipofuscin molecule, has been deemed important and was carried out.

Isolation of the Rheomelanins

The isolation was carried out in nitrogen atmosphere using deaired and nitrogen saturated solvents to avoid any oxidation. Eight 0.5-ml heparinized plasma samples were spotted on four Whatman No. 17 preparative chromatography paper strips. Each strip was 54 cm long and approximately 8 cm wide. These strips had been previously washed with distilled water and with the first developing solvent system (acetone:ethanol:H_2O = 1:1:18).

After spotting, drying and developing these strips under nitrogen, the yellow-green fluorescent spot found at an R_f = 0.75 was cut off and eluted with distilled water. The obtained rheomelanins after concentration were subjected to a second chromatography on another set of four Whatman No. 17 preparative chromatograph strips prewashed with water and the second developing solvent system (isopropanol:acetone:distilled water = 3:4:13). After spotting and drying, the strips were developed under nitrogen. The yellow-green fluorescent spot was cut off and eluted again with water.

Chemical Composition Studies

In order to determine the relationship between the isolated plasma soluble fluorescent melanins and the lipofuscins, we have applied some of the histochemical tests for solid lipofuscin in tissue slices to our isolated compounds. The isolated rheomelanins were subjected to the following tests:

1. Periodic Acid-Schiff Test
2. Sudan Black B in Alcohol
3. Alternative Nile Blue Method
4. Schmorl Test
5. Chrome Alum Hematoxylin Test
6. A Long Ziehl-Neelsen Test for Acid Fast Lipofuscins
7. Protein Determination
8. Specific Mucoprotein Test
9. Phosphoric Acid-Vanillin Lipid Determination

These methods were slightly modified to be applicable to the isolated compounds on Whatman paper No. 1, silica gel on plastic sheets and on a porcelain plate. The procedure followed is described under each of the tests.

Earlier work on lipofuscins showed that they are composed of melanins, protein, and lipids.[1] The above mentioned tests are specific for all of these components except melanins, and they have been applied in the study of tissue lipofuscin. Evidence for the existence of melanin as one of the components of the plasma isolated compound is based on the fact that incubation of heparinized plasma with catecholamines or L-Dopa resulted in an increased concentration of the isolated compound which had a characteristic yellow to dark brown color.[2] In addition, these compounds showed yellow-green fluorescence in UV at 356 nm which was not shown by other solid melanins but which is characteristic of solid lipofuscins. The excitation and fluorescence ranges and maxima of our isolated compounds in distilled water are practically identical to those of solid lipofuscins in solution.[3]

(1) Periodic Acid Schiff Test (PAS) - The rheomelanin in H_2O (≈ 1.0 mg) was spotted on a piece (1.5 cm square) of Whatman No. 1 chromatography paper and on a silica gel plastic sheet (0.2 mm thickness) without fluorescent indicator (E. Merck).

After drying, the spotted samples were immersed in a 1% aqueous periodic acid solution for 3 min, washed in water, immersed into the Schiff reagent for 20 min, rinsed 3 times with the sodium sulfite, washed with water, and left to dry. A positive reaction was indicated by the formation of a magenta color on both the paper and the silica gel plastic sheet.

No magenta color was observed using a blank chromatography paper or silica gel. Phenylether was also used as a control to check on any non-specific adsorption of the color produced. Solid lipofuscin in tissue slices also gives the magenta color by this method.

Spot tests were also carried out on a porcelain plate using the same reagents as above. Two processes were followed. In the first procedure, two drops of the isolated compound ($\sim$ 0.5 mg) were placed on the plate followed by two drops of the periodic acid reagent and left to oxidize for 20 min. This was followed by 4 drops of the sulfite solution and 4 drops of the Schiff reagent. The second procedure involved the addition of the Schiff reagent before the sulfite. A magenta color was also obtained. A blank was used by substituting the isolated compound solution with 2 drops of distilled water. Although the blank runs also showed positive results in the beginning, after the solution was left to dry on the concave surface of the porcelain plate, different colors were obtained. The blank showed a metallic yellow-bluish color while the samples showed a magenta color.

The solutions for this test, namely the de Thomasi Schiff reagent, the periodic acid solution and the sodium bisulfite rinse, were prepared following the general procedure for the test described in Culling et al.[4]

(2) Long Ziehl-Neelsen Method - This is another method utilized to identify tissue lipofuscins and to characterize them as "acid fast." A modification of the general method described in Pearse[5] has been followed. The sample, the control and the blank were prepared as were those for the previous PAS test, using Whatman paper and silica gel plastic sheets. The sample, control and blank were placed in the carbol fuchsin solution for 3 hr at 60° C. They were washed with water and transferred to a 1% acid-alcohol solution. The silica gel plastic sheets with the sample showed a more intense magenta or red-violet color than the blank or the control. However, on the Whatman chromatography paper, no differences in color between the sample, blank and control could be seen.

A spot test in a porcelain plate was also carried out. One drop of a solution of the isolated compound (0.5 mg) was mixed with one drop of distilled water and one drop of carbol fuchsin solution. Phenylether and distilled water were used as control and blank, respectively. The porcelain plate was incubated at 60° C for 3 hr. The sample developed a thick film of light magenta color. Phenylether gave a thin lilac colored film, while the blank developed a dark metallic blue spot. It is well established that tissue lipofuscin produces a bright red color when treated with carbol fuchsin in solution. According to Pearse,[5] any redness is considered a positive reaction.

(3) Alternative Nile Blue Method - A modification of the general method described in Pearse[6] has been followed. The same sample, control and blank were used on silica gel plastic sheets and Whatman paper. These were soaked in the staining solution for 30 min. The staining solution was composed of 0.05% Nile blue A solution in 1% sulfuric acid. The samples, control and blank were washed with water. The sample's spot as well as those of the control and the blank was stained dark blue. Washing the paper with ethanol or acetone partially removed the color in the blank, the control and the samples. The color was retained better on the silica gel plates after washing with ethanol or acetone, with the sample's spot showing a darker blue color layer. By washing with water, the dissolved dye forms salt unions and acquires resistance to acetone washing. Thus, we get only partial discoloration.

(4) Schmorl Test - A modification of the general procedure described in Pearse[7] was followed. The ferricyanide and the ferric chloride solutions needed in this method were freshly prepared. The same samples, controls, and blanks as in the previous method were used.

The sample, control and blank papers were immersed in the ferricyanide solution for 10 min, washed with water and dried. The sample's spot was stained blue, while the control and blank did not stain this way. Solid lipofuscins in tissue slices also stain blue.

Argentaffen granules, active sulfhydyl groups, melanin and lipofuscin are known to reduce the ferricyanide to ferrocyanide, which is blue. On the other hand, the sample spot on the silica gel sheet was stained bluish-green, while the phenylether control also stained dark blue. The blank did not show any color. The blue stain with the phenylether can not be explained.

A spot test in a porcelain plate was slso done. The sample developed a green color and turned blue later, while the control and the blank developed a green color which changed to blue overnight. This might be due to a non-specific reaction occurring just by standing overnight.

(5) Sudan Black B Test - This is a lipid staining test. The general method described by Barka and Anderson[8] has been followed. The same specimens as above were immersed in the Sudan Black B staining reagent for various periods of time: 1 min, 5 min and 20 min. They were then rinsed with 70% ethanol, followed by water. On the chromatography paper, the 1-min set comprised of sample, control and blank showed no difference in color. After 5 min and 20 min of staining, the sample developed a dark blue color. The control and blank were unchanged.

On silica gel plates, only the sample was stained dark blue after 5 min or 20 min of immersion in the staining solution.

Lipids and lipoproteins are known to stain black or blue in this method.

On the spot test, the sample turned black, which color changed to dark blue in a couple of days. The control showed a purplish dark blue color, which color turned brown in a couple of days. The blank showed a black thin film.

(6) Chrom. Alum Hematoxylin Test - The general method for this test is described in Pearse.[9] The reagents were prepared by following the published procedure. The same sample, control and blank were used as used before. The paper and the silica gel plastic sheets were transferred into the acid permanganate solution for 2 min, and then bleached by immersion in 1% oxalic acid for 1 min. They were then washed with water and stained in chrom. alum hematoxylin reagent for 10 min, and rinsed with water.

Only the sample was stained dark blue. The phenylether control and the blank were stained gray. Solid lipofuscin in tissue slices also stains dark blue with this staining method.

A spot test was done by adding to the sample (1 mg) one drop each of distilled water, acid potassium permanganate, 1% oxalic acid, and chrom. alum hematoxylin, and mixing. The sample developed a purplish chocolate color which, upon drying, showed a dark blue color. The control showed a brown color and the blank showed a dark gray color.

(7) Protein Determination - Since lipofuscins are reported to contain protein, it was important to do a total protein determination on our isolated sample. The general Lowry protein determination method has been followed.[10] This method showed a relatively high protein content by the development of a dark blue color. No quantitation has been done on this protein content.

(8) Specific Mucoprotein Test - The procedure described by Tietz[11] using electrophoresis and staining has been followed. A concentrated solution of the isolated compound was used. The band obtained by electrophoresis and located around the middle of the cellulose polyacetate strip gave purple color with Schiff's stain, indicating the presence of mucoproteins in the isolated sample.

(9) Phosphoric Acid-Vanillin Lipid Determination - The procedure described by Tietz[12] has been followed. A concentrated solution of our isolated compound has been used. A pink color was obtained. This method will be used quantitatively to determine the percent of total lipid content of our isolated compound, at a later stage.

Results and Discussion

The general modified histochemical tests for identification of lipofuscins have been applied to our isolated compound. The bulk of histochemical research is consistent with the concept that lipofuscin is the end product of progressive protein digestion and autoxidation of unsaturated lipids, with condensation of the resulting oxidized compounds into solid polymers of increasing insolubility. It seems that the main constituents of tissue lipofuscin are lipid fractions that amount to 20-50% of the dry weight of the pigment, 30-60% of the proteins and 9-20% of a highly pigmented and hydrolysis-resistant material which is believed to be a melanin-like substance mixed with peroxidized lipids and materials formed by cross-linking between lipids and proteins.[13,14]

At present, we have little idea of the significance of the various tests which are positive in the case of our isolated compound. The positive periodic acid Schiff test does not necessarily mean the presence of carbohydrates or glycolipids. Aldehydes can be produced from non-carbohydrate containing unsaturated phosphatides, by the action of periodic acid. Until much work has been done upon the problem, we can say only that, at a certain intermediate stage in the oxidation of lipids, Schiff positive groups are revealed by brief oxidation with periodic acid.

The quality of acid-fastness has been determined, using the Long Ziehl-Neelsen method and found to be positive, which is characteristic of lipofuscin. Pearse[5] regards the presence of any redness as positive.

The Nile blue test is a method for distinguishing melanins from lipofuscins and which depends on staining with the dye and subsequent treatment with hydrogen peroxide to bleach the melanins. In this test, the method of Pearse[6] was followed; our compound stained dark blue, which is reported to be characteristic of lipofuscin.

The Schmorl test is characteristic of lipofuscin and establishes the presence of reducing groups which are believed to be present at an early stage in the oxidative process of lipids. Prussian blue color or bluish green was obtained with our isolated compound, which is due to the formation of ferroso-ferric ferricyanide pigment, which is characteristic of melanin, argentaffin granules and lipofuscin, as well as tissue components containing active sulfhydryl groups.

The Sudan Black B test is a general lipid staining method. A positive test was obtained, indicating the presence of lipids in our compound. Quantitative determination of the lipid content will be carried out at a later time. The phosphoric acid Vanillin reaction has also been done for the total lipid determination, and a characteristic pink color was obtained.

The presence of proteins has been determined, following the Lowry method.[10] No quantitation has been done as yet, using this method.

A specific mucoprotein test using electrophoresis was done. This test was positive for the presence of mucoproteins, while there are still conflicting reports about the presence of carbohydrates as part of lipofuscin. Therefore, the positive result might be due to an impurity. Further investigation in this area is necessary.

Concluding Remarks

Based on all of the above mentioned tests, it can be concluded that our isolated compounds contain lipids and proteins. The fluorescence exhibited seems to ally the compounds to the lipofuscins which are, in part, melanins. In addition, the different positive tests obtained (the periodic acid Schiff test, the Long Ziehl-Neelsen test, the Nile blue test, the Schmorl test and the chrom. alum hematoxylin test) indicate another strong relationship with tissue lipofuscins.

Further experiments and investigations on the structure and content of the isolated compounds are necessary.

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Plasma soluble fluorescent compounds termed rheomelanins have been isolated using paper chromatography. The fluorescent characteristic of these compounds ally them with tissue lipofuscins. Chemical composition studies using modified tissue lipofuscin histochemical tests were conducted. In addition, protein and lipid determination tests were done. All of these tests seem to indicate a strong relationship with the lipofuscins. Further experiments and investigations on the structure and content of these compounds are necessary.

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